

## Nonhypoxic Pathway Mediates the Induction of Hypoxia-inducible Factor 1 $\alpha$ in Vascular Smooth Muscle Cells\*

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Hypoxia-inducible factor-1 (HIF-1) controls the expression of a number of genes such as vascular endothelial growth factor (VEGF) and Erythropoietin in low oxygen conditions (hypoxia). VEGF is strongly induced at both the mRNA and protein expression level by a number of hormones and growth factors in vascular smooth muscle cells (VSMC) independently of the oxygen environment. However, the role of HIF-1 $\alpha$  in this induction has not been studied. We report here that HIF-1 $\alpha$  protein levels are strongly increased by fetal calf serum in quiescent VSMC. More interestingly, Angiotensin II (Ang II), thrombin, platelet-derived growth factor, and other hormones can also increase HIF-1 $\alpha$  in VSMC to levels that are substantially more elevated than the hypoxic treatment. HIF-1 $\alpha$  induced by Ang II is located in the nucleus, binds to the hypoxic response element, and is transcriptionally active. The induction of HIF-1 $\alpha$  by hormones is mediated through the production of reactive oxygen species (ROS), since it can be blocked by the ROS inhibitors, diphenyleneiodonium and catalase. Finally, strong induction of VEGF mRNA by Ang II can also be inhibited by these ROS inhibitors. These results implicate HIF-1 $\alpha$  and HIF-1-dependent transcriptional activity in the induction of VEGF expression after agonist stimulation and define novel hypoxia-independent mechanisms that should play a major role in vascular remodeling.

The growth of new blood vessels is termed angiogenesis. Angiogenesis occurs in wound and fracture healing, arthritis, cardiovascular and cerebral ischemia, and most types, if not every type, of cancer known in humans. These events share a common characteristic of occurring in a hypoxic environment.

A major mediator of angiogenesis is vascular endothelial growth factor (VEGF)<sup>1</sup> (1–4). Transcriptional up-regulation

has been shown to play a major role in the induction of the VEGF gene, an action mediated by the specific binding of the hypoxia-inducible factor-1 (HIF-1) to the hypoxic response element (HRE). The HIF-1 transcription factor is a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$  (5). Each subunit contains an N-terminal basic helix-loop-helix domain, responsible for heterodimerization and DNA binding. Each subunit also contains a PAS (Per, ARNT, Sim) motif, which is found in a number of transcription factors including the *Drosophila* proteins Period, Single-minded, and Trachealless, as well as mammalian proteins such as AHR (aryl hydrocarbon receptor). HIF-1 $\beta$  was identified as being a previously described member of this family, the ARNT (aryl hydrocarbon receptor nuclear translocator) protein (5). C-terminal transactivation domains can be found on both HIF-1 $\alpha$  and HIF-1 $\beta$  (6–9). The mouse HIF-1 $\alpha$  gene knockout (HIF-1 $\alpha$ <sup>−/−</sup>) has clearly shown the irreversible role that HIF-1 $\alpha$  plays in neovascularization (10–12). HIF-1 $\alpha$ <sup>−/−</sup> embryos showed certain lacks and abnormalities in vessel formation. Similar defects have also been observed in VEGF knockout mice (13, 14).

While the HIF-1 $\beta$  protein is readily found in all cells, HIF-1 $\alpha$  is virtually undetectable in normal oxygen conditions. Studies have shown that, in these conditions, HIF-1 $\alpha$  is rapidly degraded by the ubiquitin-proteasome system (15–17). While hypoxia has been shown to be the ubiquitous inducer of HIF-1 $\alpha$  in all cells tested, other stimuli, such as insulin, insulin-like growth factors 1 and 2, and EGF, have also been shown to increase HIF-1 $\alpha$  protein levels in certain cell types (18–20). These stimuli are also able to induce VEGF expression in an HIF-1-dependent manner.

In vascular smooth muscle cells (VSMC), a range of different extracellular receptor agonists have been shown to induce VEGF expression, including angiotensin II (Ang II) and platelet-derived growth factor (PDGF) (21, 22). However, the status of HIF-1 $\alpha$  in these conditions has not been studied. In this work, we show that HIF-1 $\alpha$  levels in VSMC are strongly increased in normal oxygen conditions when cells are stimulated with the cell surface receptor agonists Ang II, thrombin, and PDGF. Induced HIF-1 $\alpha$  is localized in the nucleus, binds to HRE, and is transcriptionally active. Our results suggest that the increase of HIF-1 $\alpha$  protein levels is mediated through the production of reactive oxygen species (ROS). Our results clearly demonstrate that hypoxia is not the only major player in HIF-1 $\alpha$  induction and that this pathway, for the moment specific to VSMC, should play a major role in vascular VEGF production and angiogenesis.

### EXPERIMENTAL PROCEDURES

**Materials and Plasmids**—Angiotensin II, thrombin, 5-hydroxytryptamine (5-HT), diphenyleneiodonium chloride, and catalase from *Aspergillus niger* were from Sigma. PDGF and FGF were from Pepco Tech Inc. Anti-HIF-1 $\alpha$  antiserum 2087 was raised by our laboratory in rabbits immunized against the last 20 amino acids of the C terminus of

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<sup>1</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; HRE, hypoxic response element; VSMC, vascular smooth muscle cells; Ang II, angiotensin II; FCS, fetal calf serum; PDGF, platelet-derived growth factor; FGF-2, fibroblast growth factor-2; DPI, diphenyleneiodonium; 5-HT, 5-hydroxytryptamine; EGF, epidermal growth factor; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

human HIF-1 $\alpha$ . Monoclonal anti-HIF-1 $\alpha$  antibody and polyclonal anti-HIF-1 $\beta$  antibody were from Novus Biologicals (Littleton, CO). Monoclonal anti-phospho-p44/p42 MAPK antibody and PD 98059 were from New England Biolabs or Sigma. U0126 and horseradish peroxidase-coupled anti-mouse and anti-rabbit antibody were from Promega. Ly294002 was obtained from Alexis Corp. The PRE-tk-LUC reporter construct was a kind gift from Steven L. McKnight (University of Texas). pcDNA3-HA-DN-HIF-1 $\alpha$  was generated by internal digestion of pcDNA3-HA-HIF-1 $\alpha$  (23) with *EcoRI* and subsequent religation.

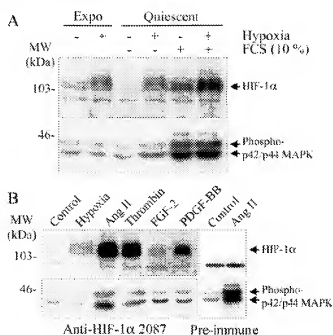
**Cell Culture**—VSMC were isolated from the thoracic aortas of 6-week-old male Harlan Sprague-Dawley rats by enzymatic dissociation (24). Cells were cultured in Dulbecco's modified Eagle's medium containing 7.5% fetal calf serum (FCS), penicillin (50 units/ml), and streptomycin (50  $\mu$ g/ml) (Life Technologies, Inc.) in a humid atmosphere (5% CO<sub>2</sub>, 95% air). Cells were serially passaged upon reaching confluence, and all experiments were performed on passages 3–10. Quiescent cells were obtained by total deprivation of FCS for 16–20 h. Pretreatment of cells with different compounds was performed 30 min prior to stimulation. Hypoxic conditions were obtained by placing the cells in a sealed "Bug-Box" anaerobic workstation (Ruskin Technologies, Leeds, United Kingdom/Jouan, Saint Herblain, France). The oxygen levels in this workstation were maintained at 1–2% with the residual gas mixture containing 93–94% nitrogen and 5% carbon dioxide.

**Western Blot Analysis**—Confluent cells were lysed in 2 $\times$  Laemmli sample buffer. Protein concentration was determined with the use of the Lowry assay. 30  $\mu$ g of whole cell extracts were resolved in SDS-polyacrylamide gels (7.5%) and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.). Proteins of interest were revealed with specific antibodies as indicated (1:1000 dilution). The bands were visualized with the ECL system (Amersham Pharmacia Biotech).

**Immunofluorescence Studies**—Cells grown on glass coverslips were fixed using 3% paraformaldehyde for 20 min followed by permeabilization with 0.1% Triton X-100 for 10 min. Coverslips were then incubated with anti-HIF-1 $\alpha$  antiserum or preimmune serum from the same animal (1:3000). Streptavidin-coupled anti-rabbit antibodies were added for 45 min followed by biotin-coupled Texas Red. Immunofluorescence was then analyzed with a Leica DM-R microscope equipped with a DC-100 digital camera.

**Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays were performed on VSMC nuclear extracts using a <sup>32</sup>P-labeled oligonucleotide containing the wild type HIF-1 binding site (25). The sense strand sequence of the oligonucleotide is 5'-GCCCTACGTGCT-GTCTCA-3'. <sup>32</sup>P-labeled oligonucleotides were generated by 5' end labeling by T4 polynucleotide kinase (New England Biolabs) with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Pharmacia Biotech). Preparation of nuclear extracts was performed as described previously (25). Binding reactions were carried out in a total volume of 30  $\mu$ l containing 10  $\mu$ g of nuclear extracts and 0.1  $\mu$ g of calf thymus DNA (Sigma) in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol. Labeled oligonucleotide probe (40,000 cpm; 0.25 ng) was added and incubated for 30 min at 4  $^{\circ}$ C. For competition experiments, a 100-fold excess of unlabeled wild-type of mutant (sense strand sequence of mutant oligonucleotide: 5'-GCCCTAAAGCT-GTCTCA-3') annealed oligonucleotide was added prior to the addition of the labeled probe. Supershift experiments were performed with either anti-HIF-1 $\alpha$  antiserum 2087, preimmune antiserum from the same animal, monoclonal anti-HIF-1 $\alpha$  antibody, or polyclonal anti-HIF-1 $\beta$  that was added after the addition of the labeled probe. DNA-protein complexes were resolved on native 6% polyacrylamide gels in 0.3 $\times$  TBE (1 $\times$  TBE: 89 mM Tris-HCl, 89 mM boric acid, and 5 mM EDTA) at 4  $^{\circ}$ C. Gels were then dried and analyzed by autoradiography.

**Transient Transfection and Luciferase Assay**—1  $\mu$ g/well of reporter plasmid was used along with 100 ng/well of cytomegalovirus  $\beta$ -galactosidase as a control for transfection efficiency. Transfection of VSMC was performed by using the Superfect transfection reagent (Qiagen) at a 1:5 DNA/reagent ratio. For dominant negative experiments, cells were transfected with 4  $\mu$ g of the pcDNA3-HA-DN-HIF-1 $\alpha$  construct, and in control cells, the same concentration of parental pcDNA3 vector was added. At 3 h post-transfection, cells were washed with medium and FCS was added. At 12 h post-transfection, cells were deprived of FCS for 16 h. Stimulation with Ang II and hypoxia was performed for 18 h. Cells were then washed twice with cold phosphate-buffered saline, and luciferase assays were performed as follows. Cells were lysed in a lysis buffer (25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N',N''-tetraacetic acid, 10% glycerol, and 1% Triton X-100) for 15 min at room temperature, and the lysate was cleared by centrifugation. The luciferase assay was performed in a



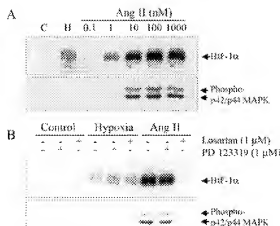
**Fig. 1. HIF-1 $\alpha$  induction by hypoxia, FCS, hormones, and growth factors in VSMC.** *A*, exponentially growing (Expo) and quiescent VSMC were maintained under control (21% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions for 4 h. The addition of 10% FCS to quiescent cells was done for the same time periods. *B*, quiescent VSMC were maintained under control conditions in the presence or the absence of either Ang II (100 nM), 1 unit/ml thrombin, 1 ng/ml FGF-2, or 10 ng/ml PDGF-BB for 4 h. For hypoxia, quiescent cells were maintained under hypoxic conditions (1% O<sub>2</sub>) for 4 h. Whole cell extracts (30  $\mu$ g) were resolved by SDS-PAGE (7.5% gel) and immunoblotted using either an anti-HIF-1 $\alpha$  antiserum, preimmune serum from the same animal, or an anti-phospho-p44/p42 MAPK monoclonal antibody.

buffer containing 20 mM Tricine, 1.07 mM (MgCO<sub>3</sub>)Mg(OH)<sub>2</sub>·5H<sub>2</sub>O (Sigma), 2.67 mM MgSO<sub>4</sub>, 3.1 mM EDTA, 33.3 mM dithiothreitol, 270 mM coenzyme A (Sigma), 470  $\mu$ M beetle luciferin (Promega), and 500  $\mu$ M ATP.  $\beta$ -Galactosidase activity was evaluated with the use of the Galacto-Light Chemiluminescent Reporter Assay kit from Tropix. Results were quantified with a MicroBeta TRILUX luminescence counter (Wallac). Results are expressed as a ratio of luciferase activity over  $\beta$ -galactosidase activity.

**Northern Blot Analysis**—Confluent cells were lysed, and RNA was isolated with RNA Instapure (Eurogentec). RNA was resolved on agarose/formaldehyde gels, was transferred to Hybond N<sup>+</sup> nylon membrane (Amersham Pharmacia Biotech), and was hybridized with a radioactive cDNA probe comprising the total coding sequence of the mouse VEGF gene. Ethidium bromide staining was used as a control to verify gel loading.

## RESULTS

**HIF-1 $\alpha$  Induction in VSMC in Normal Oxygen Conditions**—In all cell lines tested, exposure of cells to hypoxia rapidly increased HIF-1 $\alpha$  cellular protein levels. This was also the case for VSMC, since exponentially growing cells showed a strong induction of HIF-1 $\alpha$  when incubated for 4 h in 1% oxygen (Fig. 1A). However, the level of HIF-1 $\alpha$  protein expression in VSMC under normal oxygen conditions appears to be elevated in comparison with other cell lines (23). In FCS-deprived cells, the expression of HIF-1 $\alpha$  protein in normoxia was undetectable (Fig. 1A). Interestingly, when FCS-deprived VSMC were restimulated with 10% FCS for 4 h, HIF-1 $\alpha$  protein expression was strongly induced to a level that was equivalent to the increase seen in hypoxic conditions (Fig. 1A). When VSMC were stimulated with 10% FCS in hypoxic conditions, the level of HIF-1 $\alpha$  induction was additive for both stimuli. These results suggest that components of FCS are inducing HIF-1 $\alpha$  and that hypoxia and FCS increase HIF-1 $\alpha$  protein expression levels through different mechanisms. FCS contains



**FIG. 2. HIF-1 $\alpha$  induction by Ang II in VSMC.** A, quiescent VSMC were maintained under control conditions (C), under hypoxic conditions (H, 1% O<sub>2</sub>), or in the presence of the indicated concentrations of Ang II for 4 h. B, quiescent cells were pretreated for 15 min with losartan (1  $\mu$ M) or PD 123319 (1  $\mu$ M) and maintained under control conditions, under hypoxic conditions (1% O<sub>2</sub>), or in the presence of Ang II (100 nM) for 4 h. Total cell extracts (30  $\mu$ g) were resolved by SDS-PAGE (7.5% gel) and immunoblotted using an anti-HIF-1 $\alpha$  antiserum or an anti-phospho-p42/p44 MAPK monoclonal antibody.

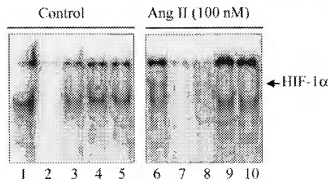
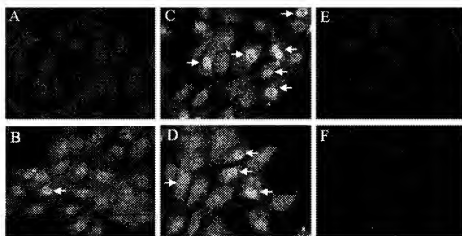
a number of hormones and growth factors that can stimulate cell surface receptors. Receptor agonists such as Ang II, thrombin, and PDGF have been shown to induce a robust increase in VEGF mRNA levels in VSMC. Since HIF-1 $\alpha$  is a major mediator of VEGF up-regulation, we wanted to evaluate the effect of the agonists on HIF-1 $\alpha$  induction. When PCS-deprived VSMC were treated for 4 h with these compounds, a strong induction of HIF-1 $\alpha$  protein expression could be observed (Fig. 1B). Stimulation of cells with serotonin (5-HT) also strongly increased HIF-1 $\alpha$  protein in this cell model (see Fig. 8B), and a weak but detectable increase could be observed with EGF-2. To confirm the specificity of the 2087 antiserum against HIF-1 $\alpha$ , we hybridized the same samples with preimmune serum from the same animal. As seen in the right panel of Fig. 1B, preimmune serum detected no band above 103 kDa and only detected a nonspecific band at 85 kDa. As a control, receptor activity was verified by the evaluation of a downstream target, the phosphorylation of the mitogen-activated kinases, p42/p44 MAPK. These kinases are strongly activated in this cell system following the addition of the previously mentioned receptor agonists. The strongest inducer of HIF-1 $\alpha$  protein expression assayed here was Ang II. A 4-h stimulation with Ang II (100 nM) increased HIF-1 $\alpha$  protein expression to levels that were more elevated than cells incubated in hypoxic conditions. As was the case with PCS and hypoxia, the combination of Ang II and hypoxia caused an additive effect on HIF-1 $\alpha$  induction (results not shown). The maximal effect of Ang II was achieved at a concentration of 100 nM. However, a significant induction could be observed at concentrations of Ang II as low as 1 nM, representing a physiological concentration for this hormone (Fig. 2A). This induction was mediated through the activation of the AT1 receptor subtype, since the AT1-specific antagonist, losartan, could completely block the induction of HIF-1 $\alpha$ , while PD 123319, an AT2-specific ligand, had no effect on basal or Ang II-induced levels of HIF-1 $\alpha$  (Fig. 2B). Therefore, these results suggest that hormones such as Ang II can strongly induce HIF-1 $\alpha$  in a normal physiological situation, an action mediated via the AT1 receptor subtype.

**Ang II Activates the HIF-1 Complex—Nuclear localization of HIF-1 $\alpha$**  has been shown to be necessary for HIF-1 activity (26). We therefore wanted to determine whether agonist-induced

HIF-1 $\alpha$  is located in the nucleus. As seen in Fig. 3A, quiescent cells in normal oxygen conditions showed very little HIF-1 $\alpha$  immunoreactivity. When cells are incubated in hypoxic conditions, an increase in HIF-1 $\alpha$  protein expression can be seen in the nucleus (Fig. 3B). When cells are stimulated with Ang II, a number of cells show a strong nuclear signal (Fig. 3C). A similar result is seen after stimulation with thrombin (Fig. 3D). These results show that agonist-induced HIF-1 $\alpha$  is localized to the nucleus. To be active, HIF-1 $\alpha$  must then form the HIF-1 transcription complex with HIF-1 $\beta$  and bind to the specific HRE DNA sequence. Therefore, we evaluated HIF-1 DNA binding activity with electrophoretic mobility shift assay experiments. When an 18-base pair oligonucleotide probe containing the HIF-1 binding site was incubated with nuclear extracts from Ang II-stimulated VSMC, a number of protein-DNA complexes were increased (compare lanes 1 and 6, Fig. 4). The identity of HIF-1 in these complexes was confirmed by competition experiments and the use of specific antibodies. An 100-fold excess of unlabeled wild-type oligonucleotide competed with the probe for the binding of HIF-1 (lane 7), whereas a 100-fold excess of an oligonucleotide containing a 3-base pair substitution in the HIF-1 binding site did not compete for binding (lane 8). Two purified and commercially available antibodies against HIF-1 $\alpha$  and HIF-1 $\beta$  disrupted the probe DNA-HIF-1 complex (lanes 9 and 10, respectively). Polyclonal antiserum 2087 raised against HIF-1 $\alpha$  also disrupted the probe DNA-HIF-1 complex, while the corresponding preimmune antiserum had no effect (results not shown). These results demonstrate that HIF-1 $\alpha$  protein induced by Ang II can form the HIF-1 complex with HIF-1 $\beta$  and bind the HRE sequence. We then evaluated whether HIF-1 induced by Ang II is transcriptionally active with the use of a luciferase reporter assay. VSMC were transiently transfected with a luciferase reporter gene (PRE-tk-Luc) driven by three 50-base pair sequences containing the HRE from the erythropoietin gene (27) and then stimulated with hypoxia and/or Ang II. As shown in the upper panel of Fig. 5, a 4-fold induction of reporter activity was attained after an 18-h incubation period in 1% oxygen. For the same time period, Ang II increased reporter activity to a level significantly higher than that elicited by hypoxia (11-fold over basal levels). As was the case in Western blot experiments (Fig. 1), the combination of both stimuli was additive (15.7-fold increase in luciferase activity). These results demonstrate that HIF-1 $\alpha$  induced by Ang II is indeed active and suggest again that hypoxia and Ang II induce HIF-1 $\alpha$  through distinct mechanisms. Since Ang II has been shown to induce VEGF mRNA, the activity of the VEGF promoter in the same conditions was also measured. To perform these experiments, we used the previously described luciferase reporter plasmid driven by a SP1/AP2-mutated and hence p42/p44 MAPK-insensitive VEGF promoter that still responds to HIF-1 (28). The transfection of this construct in VSMC followed by 18 h of hypoxia increased reporter gene activity by 1.8-fold (Fig. 5, lower panel). As with the HRE construct, Ang II increased reporter activity to a level that surpassed hypoxia (3.7-fold over basal levels). Again, both stimuli together produced an additive effect (5.1-fold). To further evaluate the involvement of HIF-1 in mediating Ang II-stimulated transcriptional activation of reporter genes containing the VEGF hypoxia response element, we used a dominant negative form of HIF-1 $\alpha$ . The pcDNA3- $\Delta$ HA-DN-HIF-1 $\alpha$  encodes a form of HIF-1 $\alpha$  lacking the C-terminal transactivation domains. DN-HIF-1 $\alpha$  can heterodimerize with HIF-1 $\beta$  and inhibit HRE-driven reporter genes (29).<sup>2</sup> VSMC were cotransfected with the mutated VEGF reporter plasmid and the

<sup>2</sup> E. Gonthé and J. Pouyssegur, unpublished results.

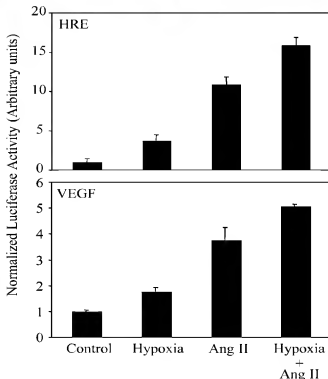
**Fig. 3. Nuclear localization of HIF-1 $\alpha$  following hormone stimulation.** Quiescent VSMC were maintained under control conditions (A), hypoxic conditions (1% oxygen) (B), or in the presence of Ang II (100 nM) (C) or thrombin (1 unit/ml) (D) for 4 h and analyzed by immunofluorescence using an anti-HIF-1 $\alpha$  antiserum. The arrows indicate strong nuclear localization. VSMC under control conditions and stimulated with Ang II were also probed with preimmune serum from the same animal to evaluate nonspecific signal (E and F, respectively).



**Fig. 4. Ang II induces an HIF-1-DNA complex.** Quiescent VSMC were incubated in the presence or the absence of Ang II (100 nM) for 4 h. Nuclear extracts were prepared as indicated under "Experimental Procedures." Nuclear protein (10  $\mu$ g) was incubated in the presence of a  $^{32}$ P-labeled oligonucleotide containing the HIF-1 binding site. Competitors and antibodies were also added to the binding assays to evaluate the specificity of the protein-DNA complexes. For competition assays, unlabeled oligonucleotide (25 ng) was included in the binding reaction mixture. Lanes 1 and 6, no competitor; lanes 2 and 7, wild type oligonucleotide; lanes 3 and 8, mutated HRE oligonucleotide. For supershift assays, purified monoclonal anti-HIF-1 $\alpha$  (lanes 4 and 9) and polyclonal anti-HIF-1 $\beta$  (lanes 5 and 10) antibodies were also added to the binding reaction mixture.

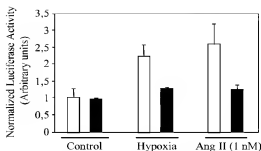
pcDNA3-HA-DN-HIF-1 $\alpha$  construct followed by incubation of cells in hypoxic conditions or in the presence of Ang II. A low concentration of Ang II (1 nM) was used, since this concentration induces HIF-1 $\alpha$  protein at levels similar to hypoxia (Fig. 2A). As seen in Fig. 6, activation of promoter activity is similar between hypoxic and Ang II stimulation (2.2- and 2.5-fold, respectively). When cells were transfected with dominant negative HIF-1 $\alpha$ , a strong inhibition of reporter activity could be observed when cells were incubated in hypoxic conditions or in the presence of 1 nM Ang II (Fig. 6, filled bars). Taken together, all of these results demonstrate that Ang II induces VEGF expression by increasing HIF-1 $\alpha$  and the HIF-1 transcription complex.

**Induction of HIF-1 $\alpha$  by Ang II Is Not Mediated through Activation of p42/p44 MAPK or Phosphatidylinositol 3-Kinase**—We next wanted to identify the mechanism by which HIF-1 $\alpha$  was induced by Ang II. Since Ang II strongly increases p42/p44 MAPK activity in VSMC, we wished to evaluate whether this pathway was implicated in the induction of HIF-1 $\alpha$  protein expression by Ang II. PD 98059 and U0126 are specific and potent inhibitors of an upstream p42/p44 MAPK kinase, MEK1. We pretreated VSMC with these compounds and evaluated HIF-1 $\alpha$  induction after Ang II stimulation. Only a slight decrease in the Ang II-increased levels of HIF-1 $\alpha$  could

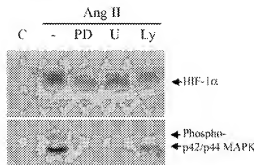


**Fig. 5. Ang II stimulates HIF-1 transcriptional activity.** VSMC ( $5 \times 10^5$  cells/well, six-well plate) were transfected with 1  $\mu$ g of either the PRE-4k-LUC construct (upper panel) or mutated VEGF-LUC (lower panel) reporter plasmid and 100 ng of an expression vector coding for  $\beta$ -galactosidase in order to normalize for transfection efficiency. 12 h after transfection, cells were deprived of FCS for 16 h. Cells were then maintained under control (21% O $_2$ ) or hypoxic conditions (1% O $_2$ ) in the presence or absence of Ang II (100 nM) for 18 h. At this point, VSMC were lysed, and luciferase and  $\beta$ -galactosidase activity were measured as described under "Experimental Procedures." Results are expressed as a ratio of luciferase activity over  $\beta$ -galactosidase activity and are representative of three similar experiments performed in triplicate.

be seen after this pretreatment (Fig. 7), while p42/p44 MAPK activity was completely blocked. This result is in accordance with that obtained in another model system, CCL39 fibroblasts (results not shown). The phosphatidylinositol 3-kinase/AKT pathway has been shown to be implicated in the induction of HIF-1 $\alpha$  protein expression (18, 30). This pathway has been shown to be activated in VSMC following short term stimulations with Ang II (31). To investigate the participation of this pathway in Ang II-mediated HIF-1 $\alpha$  protein expression, we used a potent inhibitor of this pathway, Ly294002. As was the



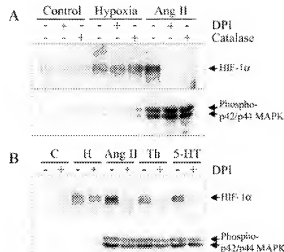
**Fig. 6. A dominant negative form of HIF-1 $\alpha$  blocks Ang II-induced VEGF promoter activity.** VSMC ( $5 \times 10^5$  cells/well, six-well plate) were transfected with 1  $\mu$ g of mutated VEGF-LUC reporter plasmid, 4  $\mu$ g of pcDNA3 (empty vector), 4  $\mu$ g of pcDNA3-HA-DN-HIF-1 $\alpha$  (filled bars), and 100 ng of an expression vector coding for  $\beta$ -galactosidase in order to normalize for transfection efficiency. 12 h after transfection, cells were deprived of FCS for 16 h. Cells were then maintained under control (21% O $_2$ ) conditions, under hypoxic conditions (1% O $_2$ ), or in the presence of Ang II (1 nM) for 18 h. At this point, VSMC were lysed, and luciferase and  $\beta$ -galactosidase activity were measured as described under "Experimental Procedures." Results are expressed as a ratio of luciferase activity over  $\beta$ -galactosidase activity and are representative of three similar experiments performed in triplicate.



**Fig. 7. Induction of HIF-1 $\alpha$  by Ang II is independent of the p42/p44 MAPK and phosphatidylinositol 3-kinase pathways.** Quiescent cells were incubated in the presence or absence (C) of Ang II (100 nM) for 4 h. For inhibitors, cells were pretreated for 15 min with PD 98059 (50  $\mu$ M; PD), U0126 (25  $\mu$ M; U), or LY294002 (50  $\mu$ M; Ly) before the addition of Ang II (100 nM) for 4 h. Total cell extracts (30  $\mu$ g) were resolved by SDS-PAGE (7.5% gel) and immunoblotted using an anti-HIF-1 $\alpha$  antiserum or an anti-phospho-p42/p44 MAPK monoclonal antibody.

case with the MAPK inhibitors, pretreatment of cells with LY294002 only had a modest inhibitory effect on HIF-1 $\alpha$  induction by Ang II. These results demonstrate that the activation of these two separate signaling cascades is unlikely to represent the major pathway by which Ang II is able to induce HIF-1 $\alpha$  in VSMC.

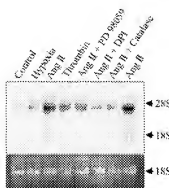
**Reactive Oxygen Species Are Necessary for Ang II-induced HIF-1 $\alpha$  Induction.** Recent evidence has convincingly shown that in VSMC, Ang II, thrombin, and PDGF can modulate the expression and activity of different elements of the NAD(P)H oxidase system (32–36). This system is responsible for strong increases in intracellular ROS levels found in these cells after growth factor and hormone stimulation. In VSMC, ROS production is essential for the activation of a number of intracellular transduction pathways (31, 37, 38). Interestingly, recent results have suggested a possible role for ROS in HIF-1 $\alpha$  induction in Hep3B cells (39). Therefore, we decided to investigate whether an increase in ROS levels was responsible for the induction of HIF-1 $\alpha$  mediated by Ang II in VSMC. The flavoprotein-containing enzyme inhibitor diphenyleneiodonium (DPI) has been widely used to evaluate the activity of this pathway. DPI has been shown to inhibit Ang II-, thrombin-, and PDGF-mediated induction of ROS production in VSMC (31, 34, 35). When cells were pretreated with 10  $\mu$ M DPI prior to stimulation with Ang II, HIF-1 $\alpha$  induction was completely



**Fig. 8. ROS inhibitors block hormone-mediated HIF-1 $\alpha$  induction.** Quiescent VSMC were maintained under control conditions (Control), under hypoxic conditions (Hypoxia; 1% O $_2$ ), or in the presence of either Ang II (100 nM), thrombin (1 unit/ml), or 5-HT (1  $\mu$ M) for 4 h. For inhibitors, cells were pretreated for 15 min with DPI (10  $\mu$ M) or catalase (1000 units/ml) prior to stimulation. Total cell extracts (30  $\mu$ g) were resolved by SDS-PAGE (7.5% gel) and immunoblotted using an anti-HIF-1 $\alpha$  antiserum or an anti-phospho-p42/p44 MAPK monoclonal antibody.

inhibited (Fig. 8A). The addition of extracellular catalase acts as an antioxidant and decreases ROS levels in the cells. The addition of 1000 units of catalase to VSMC culture medium prior to the addition of Ang II also completely blocked the induction of HIF-1 $\alpha$  (Fig. 8A). The induction of HIF-1 $\alpha$  in these cells by other hormones is also inhibited by DPI (Fig. 8B). DPI or catalase did not affect normal receptor signaling, since p42/p44 MAPK activation by the agonists was not affected by DPI or catalase. These results strongly suggest that HIF-1 $\alpha$  induction by Ang II is mediated through ROS production. It is interesting to note that in this cell model, hypoxic induction of HIF-1 $\alpha$  is not affected by treatment with DPI or catalase. These results support the data shown in Fig. 1 and 5, which suggest that hypoxia and Ang II induce HIF-1 $\alpha$  through different mechanisms.

**ROS Inhibitors Inhibit Ang II-induced VEGF mRNA Expression.** As previously mentioned, Ang II has been shown to induce VEGF mRNA expression. In Figs. 5 and 6, we showed that Ang II could activate VEGF promoter activity, through the induction of HIF-1 activity. Since ROS inhibitors blocked HIF-1 $\alpha$  induction by Ang II, we used DPI and catalase to confirm the role played by Ang II-induced HIF-1 $\alpha$  in VEGF mRNA expression. When quiescent VSMC are stimulated with Ang II, robust increase in the levels of VEGF mRNA could be detected (Fig. 9). As was the case in the luciferase assay, this increase was much higher than that elicited by hypoxic conditions. Thrombin also increased VEGF expression. More importantly, when cells were pretreated with DPI or catalase before the addition of Ang II, the ability of Ang II to stimulate VEGF expression was significantly attenuated. The addition of these compounds had no effect on VEGF mRNA levels in nonstimulated and hypoxic conditions (results not shown). Taken together with the results seen on HIF-dependent transcriptional activity, these results demonstrate that the increase of VEGF mRNA after hormone and growth factor stimulation is HIF-1-mediated. We have previously shown that strong p42/p44 MAPK activity can increase expression of VEGF in fibroblasts by modulating SP1 and AP2 binding to the promoter and by directly phosphorylating HIF-1 $\alpha$  and increasing HIF-1 transcriptional activity (23, 28). As Ang II strongly activates p42/



**FIG. 9. ROS inhibitors block Ang II-mediated VEGF mRNA expression.** Quiescent VSMC were maintained under control conditions (Control), under hypoxic conditions (Hypoxia; 1% O<sub>2</sub>), or in the presence of either Ang II (100 nM) or thrombin (1 unit/ml) for 4 h. For inhibitors, cells were pretreated for 15 min with PD98059 (50  $\mu$ M), DPI (10  $\mu$ M), or catalase (1000 units/ml) prior to stimulation with Ang II. Total mRNA was extracted and resolved on formaldehyde agarose gels. Northern blot was performed using a specific radiolabeled VEGF probe. Ethidium bromide staining was used as a control for gel loading (lower panel).

p44 MAPK in VSMC, we wanted to evaluate the contribution of this signaling pathway in Ang II-mediated increases in VEGF mRNA. Preincubation of VSMC with PD 98059 led to a modest decrease in Ang II-induced VEGF mRNA levels. This confirms that p42/p44 MAPK is partly implicated in this increase. However, in this cell system, p42/p44 MAPK appears to be mediating its effects through HIF-1, since the inhibition caused by the addition of both DPI and PD 98059 together is the same as DPI alone (results not shown). These results clearly show the key role played by HIF-1 in the induction of VEGF mRNA in VSMC.

#### DISCUSSION

Ang II, thrombin, and PDGF have all been shown to induce VEGF expression in VSMC (Refs. 21 and 22 and this study). However, no study has investigated the signal pathways involved in this effect. Previous studies have shown the major role that HIF-1 $\alpha$  plays in VEGF expression and angiogenesis (10–12, 40). It was therefore tempting to hypothesize that the ability of hormones and growth factors to up-regulate VEGF expression in VSMC was mediated through an increase in HIF-1 $\alpha$ . In addition to the classical hypoxic/cobalt/deferrioxamine-mediated induction of HIF-1 $\alpha$ , a number of agonists such as insulin, insulin-like growth factor 1, EGF and PGF, the organo-mercurial compound merasyl, and the peptide antibiotic PK39 have also been shown to induce the expression of this transcription factor (18–20, 41, 42). These studies suggest that while hypoxia remains the undisputed ubiquitous inducer of HIF-1, other factors can also modulate increases of HIF-1 $\alpha$  protein levels in a cell-specific manner. In this report, we set out to determine the role played by HIF-1 $\alpha$  in the nonhypoxic induction of VEGF in VSMC. In this cell line, we have made three significant findings: 1) hormones and growth factors can induce HIF-1 $\alpha$  in VSMC; 2) hypoxia and hormones function through two separate pathways to induce HIF-1 $\alpha$ ; and 3) hormonal and growth factor-mediated increases in HIF-1 $\alpha$  mediate VEGF mRNA expression in VSMC.

We show that Ang II, thrombin, and PDGF strongly induce HIF-1 $\alpha$  in VSMC. These effects appear to be cell-specific for the following reasons: 1) Ang II does not induce HIF-1 $\alpha$  in another cell model known to have a high level of AT1 receptors, adrenal glomerulosa cells<sup>3</sup>; 2) in endothelial cells, thrombin and PDGF

did not increase HIF-1 $\alpha$  levels; and 3) in CCL39 fibroblasts, only a small increase (10-fold lower than hypoxia) can be seen after stimulation with thrombin or PDGF.<sup>4</sup> Previous studies have shown that HIF-1 $\alpha$  induced by blocking its degradation by the ubiquitin-proteasome with specific inhibitor is transcriptionally inactive. These results suggest that other signals along with HIF-1 $\alpha$  stabilization are needed for full HIF-1 activity. After stimulation with Ang II, increased HIF-1 $\alpha$  protein levels are found in the nucleus, the HIF-1 transcription complex is formed and can bind to the HRE binding site, and HIF-1 is transcriptionally active. Therefore, Ang II receptor activation in VSMC activates all of the signals necessary for a full HIF-1 response.

These results led us to investigate the possible mechanism that is implicated in hormonal induction of HIF-1 $\alpha$ . Reactive oxygen species have been shown to be implicated in HIF-1 activity (39, 43). Different hormones and growth factors have been shown to activate the production of ROS in VSMC (31, 34, 35). It is interesting to note that VSMC specifically contain homologues to the various proteins that are similar to components of the phagocytic NAD(P)H oxidase, whose activation leads to the production of ROS (32–36). Recent research has shown that the activity of the VSMC NAD(P)H system is increased by hormones and growth factors such as Ang II, thrombin, and PDGF (32–36). The mechanism by which these compounds activate the NAD(P)H oxidase remains unclear. Cell signaling pathways in VSMC have been shown to be activated by this ROS system (31, 37, 38). DPI and the addition of external catalase have been shown to inhibit agonist-induced NAD(P)H oxidase-mediated ROS production in VSMC (31, 34, 35). We show that DPI and catalase can completely inhibit the induction of HIF-1 $\alpha$  in these same cells, which therefore implicates hormone-generated ROS in this effect. Interestingly, the hypoxic signaling pathway in these cells appears to be independent of ROS, since the inhibitors do not block the hypoxic induction of HIF-1 $\alpha$ . These results were suggested in our first experiments in VSMC, since HIF-1 $\alpha$  induction and activity were strictly additive and are in contradiction with studies that show that ROS generation is required for hypoxic response in Hep3B cells (39). However, we do see an inhibition of the hypoxic induction of HIF-1 $\alpha$  in CCL39 fibroblasts.<sup>4</sup> This suggests that this effect is specific to VSMC. We are currently evaluating the possible mechanism implicated in hypoxia-mediated increases of HIF-1 $\alpha$  in quiescent VSMC.

HIF-1 $\alpha$  is a major mediator of VEGF expression in a number of cells. Since Ang II increased the HRE- and VEGF-driven reporter activity, it seemed likely that HIF-1 $\alpha$  induction mediates VEGF expression in VSMC. Experiments with the dominant negative HIF-1 $\alpha$  construct and the finding that ROS inhibitors also block VEGF mRNA expression confirm this hypothesis. As was the case for HIF-1 $\alpha$  protein induction, hypoxic induction of VEGF in quiescent VSMC is not mediated through ROS production. This finding is in agreement with results from Wenger *et al.* (44), who have shown that hypoxic induction of VEGF mRNA expression is preserved in B lymphocytes deficient for the p22<sup>phox</sup> and gp91<sup>phox</sup> subunits of the NADPH oxidase. However, Chandel *et al.* (39) suggest that the induction of HIF-1-regulated genes during hypoxia is mediated through mitochondrial ROS produced at complex III. Again, it is possible that the effect we show is specific to VSMC. Since these cells are close to a high supply of oxygen and hormonal stimulation gives such a strong effect, VSMC may have shunted a hypoxic induction mechanism found in other cells. Evidently, these results support to the notion that hypoxia

<sup>3</sup> S. N. Poirier and D. E. Richard, unpublished observations.

<sup>4</sup> D. E. Richard and J. Pouyssegur, unpublished observations.

signal transduction is not a simple linear pathway.

In conclusion, our study provides convincing data that identify an interesting mechanism by which Ang II can induce VEGF expression in VSMC. Ang II binds to the AT1 receptor and increases ROS levels through activation of the NAD(P)H oxidase. ROS would then induce HIF-1 $\alpha$ . An active HIF-1 complex is then formed, which activate its target genes including a strong induction of VEGF. It is likely that these results have a physiological relevance given the low concentrations of Ang II that can induce HIF-1 $\alpha$ . Finally, these results shed light on the mechanisms involved in the hormonal modulation of angiogenesis.

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